AD-A175 215 AN INVESTIGATION OF THE MEMORY RESPONSE OF THE LOCAL IMMUNE SYSTEM TO SHIGELLA ANTIGENS(U) MICHIGAN UNIV ANN ARBOR D F KEREN 31 DEC 85 DAMD17-85-C-5006 1/1

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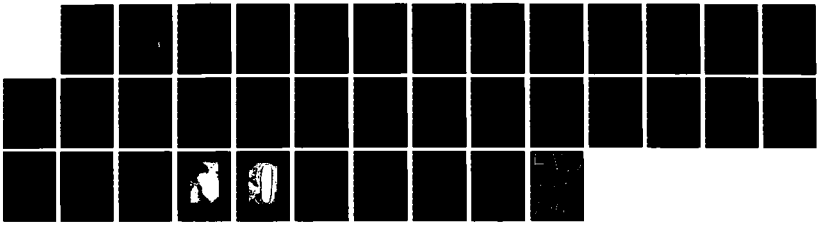

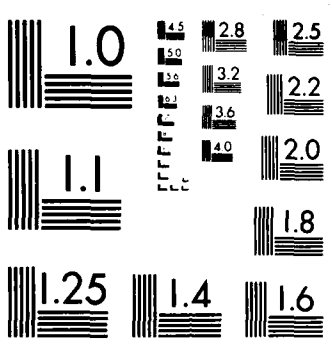

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AN INVESTIGATION OF THE MEMORY RESPONSE OF THE LOCAL IMMUNE SYSTEM TO SHIGELLA ANTIGENS

ANNUAL REPORT
DAVID F. KEREN, M.D.
DECEMBER 31, 1985

FOR THE PERIOD DECEMBER 1, 1984 - NOVEMBER 30, 1985

SUPPORTED BY

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

CONTRACT NO. DAMD 17-85-C-5006

THE UNIVERSITY OF MICHIGAN
Ann Arbor, Michigan 48109

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp Date Jun 30, 1986

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for Public Release; Distribution Unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION The University of Michigan 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Ann Arbor, Michigan 48109	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research and Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-85-C-5006
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 62770A PROJECT NO. 3M1-62770A870 TASK NO. AD WORK UNIT ACCESSION NO 023
11. TITLE (Include Security Classification) An Investigation of the Memory Response of the Local Immune System to Shigella Antigens	
12. PERSONAL AUTHOR(S) Keren, David F.	
13a. TYPE OF REPORT Annual 13b. TIME COVERED FROM 12/1/84 TO 11/30/85	14. DATE OF REPORT (Year, Month, Day) 1985, December 31 15. PAGE COUNT 34
16. SUPPLEMENTARY NOTATION	

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	13	
06	03	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
mucosal immunity, Shigella flexneri, secretory IgA, mucosal vaccine, memory response, virulence plasmid, adjuvants, gut-associated lymphoid tissue (GALT)

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The present studies center on optimizing the mucosal humoral immune response to Shigella flexneri. Using an isolated ileal loop model system in rabbits, the present work demonstrates that a hyperstimulation of the primary local IgA response can be elicited by a combination of parenteral and oral immunization. The rapid production of secretory IgA against S. flexneri could be especially useful in combating sudden outbreaks of enteric disease. Cell culture techniques are described which will be used to learn how to best stimulate cells that are committed to synthesize IgA anti-shigella lipopolysaccharide. Lastly, the role played by mucosal humoral immunity in host defense against dysentery is still unclear. We are in the process of developing both in vivo and in vitro model systems which will allow us to document the role of the anti-shigella mucosal immune response in preventing adherence, invasion and ulceration. Such assay systems are critical to predicting the utility of mucosal vaccines to a variety of enteropathogens.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia Miller	22b. TELEPHONE (Include Area Code) 301/663-7325 22c. OFFICE SYMBOL SGRD-RMI-S

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted
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SECURITY CLASSIFICATION OF THIS PAGE

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Summary

In the present studies, we have used several approaches to study ways to optimize immune responses to Shigella flexneri. Studies on maximizing the mucosal humoral immunity were performed using our chronically isolated ileal loop model in rabbits as a probe to follow the secretory immune response. A cell culture system was established which will be used to follow the production of lymphocytes committed to the development of IgG and IgA anti-shigella LPS. Several systems were evaluated for determining the functional significance of IgG and IgA anti-shigella LPS for inhibiting pathologic effects of S. flexneri. Lastly, an ultrastructural model for following the uptake of S. flexneri by specialized epithelium was begun.

Using the isolated ileal loop model system in rabbits, we have demonstrated that a hyperstimulation of the primary local IgA response can be elicited by a combination of parenteral and oral immunization. The optimal regimen consisted of an intramuscular injection of heat-killed S. flexneri followed one day later by an oral dose of live S. flexneri. Such rapid production of secretory IgA against enteropathogens could be especially useful in combating sudden outbreaks of enteric disease. The duration of this response is not yet known. However, our previous studies demonstrated that a long term mucosal memory response can be elicited by oral immunization with live S. flexneri. Since many of the vaccine strains contained the virulence plasmid, there has been concern that reversion to an invasive strain was possible. In the present studies we document that noninvasive S. flexneri which have been cured of the virulence plasmid are able to elicit this same secretory IgA memory response in intestinal secretions. This indicates that a safe, live oral vaccine could be used to stimulate the long term secretory IgA response to enteropathogens.

To document the events involved in both the hyperstimulation of the secretory IgA response and the mucosal memory response to S. flexneri, we have established a cell culture system. This has allowed us to demonstrate the presence of antigen-specific IgG and IgA immunoblasts in tissues at various times following vaccination. By using this model system in future studies, we will learn how to best stimulate cells that are committed to synthesize IgA anti-shigella LPS.

The role played by mucosal and humoral immunity to S. flexneri is still unclear at the present time. Therefore, the present studies evaluated several in vivo and in vitro model systems which examined attachment of shigella, invasion by shigella, clinical effects of shigella in rabbits and the possible interference of these effects by specific antibodies. We documented the usefulness of two of these

systems for future studies. The adherence and invasion of HeLa cells by S. flexneri proved to give a reproducible effect which allowed us to quantify an inhibitory effect of specific antisera. An in vivo system using a temporary ligature on the terminal ileum to facilitate colonization and invasion by S. flexneri was also useful. With this system, we could follow increases in peripheral blood leukocytes and histologic evidence of invasion.

Lastly, we believe that the main reason for the immunogenicity of noninvasive strains, but the requirement for viability are related to the process by which antigen is taken-up in the bowel. Other workers have recently shown that live bacteria are sampled more readily by the gut-associated lymphoid tissues (GALT) than nonviable bacteria. In our initial studies of the kinetics of the uptake of S. flexneri by the specialized epithelium over GALT we demonstrated viable S. flexneri within these cells by 30 minutes and that by 4 hours they are structurally intact and packaged into vescicles. The kinetics of this uptake, variation with viability and virulence will be correlated with the ability of those preparations to elicit a secretory IgA memory response to S. flexneri.

FOREWORD

During the course of this work, the author was greatly assisted by Roderick McDonald, Liz Struble, Scott Kern, Pam Lincoln, Lora Jo Anselmi, Joseph Waseff and Phillip Lewandowsky. The ability to perform cell culture techniques has been due to the work of Dr. John Carey.

In conducting the research described in this report, the investigator followed the "Guide for Care and Use of Laboratory Animals" prepared by The Committee on Care and Use of Laboratory Animals of The Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (NIH Publication 85-23 Revised 1985).

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INTRODUCTION

In recent years, the contributions of the mucosal immune system to host defense against a wide variety of pathogenic microorganisms have been recognized. As the mucosal immune system lies at the portal of entry for enteropathogens, it has great potential strategic importance to preventing or altering the natural course of infectious diseases of the gastrointestinal, respiratory and genito-urinary tracts. Its relative inaccessibility made the mucosal immune system difficult to study in a sequential manner.

This problem was solved in our laboratory by developing chronically isolated loops of ileum in rabbits as a probe to follow the secretory immune response of the intestine (1). Using this model, we have characterized the intestinal IgA response to cholera toxin, Shigella flexneri, Salmonella typhi, and to keyhole limpet hemocyanin (2-6). The use of such isolated ileal loops as a probe is a logical extension of the present understanding of antigen processing and lymphocyte trafficking following antigen administration. Briefly, antigen is taken-up by specialized "M" cells in the epithelium overlying Peyer's patches and the appendix (7,8). "M" cells have been recently shown by our group also to be present in isolated intestinal lymphoid follicles (9). IgA precursor B lymphocytes and regulatory T cells in the gut associated lymphoid tissues (Peyer's patches, appendix, isolated lymphoid follicles, mesenteric lymph nodes) are stimulated by this antigen. Subsequently, the IgA precursor B lymphocytes migrate to the thoracic duct, mature in the spleen (as well as other unidentified sites) and eventually return to the gut mucosa as well as to other mucosal surfaces (10-13). In some species, considerable IgA is transported into the bile which results in enhanced mucosal protection in the upper small intestine (14,15).

Our previous work has documented several major features of the local immune response relative to potential vaccine programs. First, an intestinal secretory IgA response to S. flexneri is best elicited by oral rather than parenteral immunization (6). Second, a local secretory IgA memory response can be elicited by oral immunization with live but not killed S. flexneri (16). Third, although the S. flexneri need to be live, noninvasive strains such as Shigella X16 (hybrid of E. coli and S. flexneri) and S. flexneri strain 2457-0 could both elicit local secretory IgA memory responses in intestinal secretions (17). Lastly, the major lesions seen in rabbits given pathogenic strains of S. flexneri were most frequently seen over Peyer's patches and isolated lymphoid follicles, implying that these may serve as the major site of invasion and (or) initial antigen processing (3).

The presently reported studies have concentrated on five major goals. 1) The role of adjuvants in enhancing the primary secretory IgA response of the intestine to enteropathogens was documented. 2) We show that nonpathogenic strains of S. flexneri can prime animals for a mucosal memory response. 3) Cell culture techniques were established which will allow us to follow the sensitization of antigen-specific B lymphocytes and their maturation to preferentially produce IgA rather than other immunoglobulin classes. 4) Four model systems have been explored to examine the functional significance of secretory IgA to enteropathogens. Two of these systems have given consistent results in vivo for following the clinical course of the experimental shigella infections in rabbits. 5) The early events involved in processing of shigella by specialized surface epithelial cells, "M" cells, are being studied. Overall, the present work lays the basis for understanding both how to stimulate optimally the local immune response to mucosal pathogens and how to estimate whether the responses elicited would have a protective effect against the infection.

Methods

Preparation of Chronically Isolated Ileal Loops. The surgical creation of ileal Thiry-Vella loops in rabbits has been described in detail previously (1). Briefly, 3 kg New Zealand white rabbits are anesthetized with xylazine and ketamine. A midline abdominal incision is made and the terminal ileum is identified. Twenty centimeters of ileum containing a Peyer's patch is isolated with its vascular supply intact. Silastic tubing (Dow-Corning) is sewn into each end of the isolated segment. The free ends of the tubing are brought out through the midline incision and are tunnelled subcutaneously to the nape of the neck where it is exteriorized and secured. Intestinal continuity is restored by an end-to-end anastomosis and the midline incision is closed in two layers.

Each day about 2-4 ml of secretions and mucus that collect in the ileal loops are expelled by injecting 20 ml of air into one of the silastic tubes. The slightly opaque, colorless fluid and mucus expelled from the tubing is studied for specific immunoglobulin content. A subsequent flush with 20 ml of sterile saline helps to remove adherent mucus. This saline is then removed by repeated gentle flushes of air. With proper daily care, > 90% of our rabbits have completed experiments lasting 2 months.

Enzyme-linked Immunosorbent Assay (ELISA). Microtiter wells are coated with a solution containing *S. flexneri* lipopolysaccharide (LPS) (Westphal preparation). Immediately prior to testing serum samples or loop secretions, the antigen solution is removed and the wells are washed with a phosphate-buffered saline solution (PBS) containing .05% Tween 20 (PT). The fluid to be assayed is diluted in the PT buffer and incubated in the coated and uncoated wells (the latter to control for nonspecific adsorption) for 4 hours on a horizontal rotary shaker. The plates are washed with PT and incubated with either alkaline phosphatase-conjugated sheep anti-rabbit IgA or sheep anti-rabbit IgG (both are isotype specific affinity column purified in our laboratory using methods previously described [18]). After an incubation of 4 hours, the wells are again washed with PT and the substrate reaction is carried out with p-nitrophenyl phosphate in carbonate buffer pH 9.8. Kinetics of the enzyme-substrate reaction are extrapolated to 100 minutes. The OD 405 nm (read on a Titertek Microelisa Reader) of the uncoated wells is subtracted from that of the coated wells. Specific IgG and IgA standards are processed on each plate with the unknown fluids as previously described (18,19).

The data are presented as geometric means, since others have noted that this better reflects the logarithmic kinetics of the local immune response after immunization (20). These were calculated by using the log10 of each value for each

rabbit to determine the mean, standard deviation, and standard error of the mean. For each days result, to determine the variance, the log10 standard error of the mean was added and subtracted from the mean log of specific immunoglobulin activity; antilogs of these three values were then obtained to give the geometric mean with an upper and lower limit of variance about that mean. Significance was calculated using Student's T test.

Antigen and Adjuvant Preparations Used. Four antigen preparations were employed in these studies: 1) live S. flexneri M4243 (which can invade intestinal mucosa and persist in the intestine), 2) live Shigella X16 (which invade the intestinal mucosa, but do not persist), 3) live S. flexneri 2457-0 (which do not usually invade, but which possess a virulence plasmid), 4) S. flexneri M4243A1 (which have been cured of the virulence plasmid and are unable to invade). All strains are tested for invasion using the Sereny test. The Sereny test is performed weekly on strain M4243 to assure the invasive activity of this strain for adhesion and invasion studies. Two adjuvant preparations were used in the present studies. The proposed mucosal adjuvant Avridine was kindly provided by Dr. Keith Jensen. For parenteral studies, complete Freund's adjuvant was used.

Electron Microscopy. All tissues for electron microscopy were minced with a fresh scalpel blade and fixed in 3% glutaraldehyde and PBS for 2 hours. Postfixation was carried out in 2% S-collidine-buffered osmium tetraoxide. The tissue was stained with 2% uranyl acetate and embedded in polybed. Sections were made on a Sorvall MT2 ultramicrotome and stained with Reynold's lead acetate. Thick sections (1 micron) were stained with toluidine blue. Electron microscopy was carried out on a Zeiss 109 fiberoptic transmission electron microscope.

In Vitro Assays for Adherence of S. flexneri. Two assay methods were explored. The first involved the use of whole ileum and colon tissue sections from rabbits. The second used a HeLa tissue culture line.

1. Sections of ileum and colon were opened and the contents gently expelled with saline moistened gauze. The bowel was cut into segments 1-2cm. Following a brief rinse in two separate beakers of saline, the segments were placed into a tared cup containing 10 ml of modified Krebs Ringer Solutions (KRS) and weighed. The segments were placed in a solution containing S. flexneri M4243 of varying concentrations (indicated) for 20 minutes at 37 C with gentle shaking in a water bath. Thereafter, each segment was dipped 4 times (15 seconds each) in successive beakers of saline and placed into a cup containing 10 ml modified KRS. In each experiment, 1 segment was frozen to examine the location of the adhering shigella. Remaining segments were individually

homogenized for 15 seconds using the Virtis blender. The homogenate was poured into another cup and allowed to settle for 10 minutes. Both the homogenate and the supernatant fluid (from the initial incubation) were used for plating on MacConkey agar. Viable counts of the homogenates were determined by plating 0.1 ml of serial dilutions onto MacConkey agar. The percentage of viable shigella adhering to the tissue sections was calculated by dividing the number of shigella per ml of KRS present after incubation by 100. Giemsa stains of the frozen sections were made to determine the location of the shigella on the tissue (serosal vs. epithelial surface).

2. HeLa cells were grown in Minimal Essential Medium (MEM) with 10% fetal calf serum containing 1% antibiotic-antimycotic (Gibco Laboratories). Sixty thousand HeLa cells were grown overnight to form a contiguous monolayer on Lab-Tek tissue culture chamber slides (Miles Scientific). Following a gentle rinse with MEM, S. flexneri M4243 were added to the chamber in the indicated concentrations and allowed to incubate for 30 minutes at 37 C (infection period). After another brief rinse with MEM, they were allowed to incubate for 3 hours at 37 C (multiplication period). Slides were fixed in ethanol and stained with Giemsa. For each preparation, 100-200 HeLa cells were counted and rated for bacterial involvement.

0 = no rods attached
+/- = 1 rod/HeLa cell
1+ = 2-4 rods/HeLa cell
2+ = 5-10 rods/HeLa cell
3+ = > 10 rods/HeLa cell

In Vivo Assays for Adherence of S. flexneri. Two assay systems were used. The first involved the use of intact rabbits with an oral challenge route. The second involved the surgical creation of a ritard system and direct inoculation of bacteria into the jejunum.

1. Rabbits were fasted for 48 hours prior to challenge with bacteria. Oral doses of S. flexneri M4243 were given (via orogastric tube) to rabbits anesthetized by xylazine and ketamine. For two days prior to challenge and 2-4 days after challenge, animals had rectal temperatures recorded at 9 AM and 3 PM and total white blood cell count and differentials were obtained. Daily cultures of stool were made on MacConkey's agar. At time of sacrifice, histologic sections of jejunum, ileum (including a Peyer's patch) and colon were routinely obtained. These were graded for inflammation and bacterial invasion. Also, cultures on MacConkey agar were made of the jejunal, ileal and colonic contents.

2. Rabbits were fasted for 24 hours and anesthetized with xylazine and ketamine. The abdomen was opened under aseptic conditions and sterile umbilical tape was used to

create a slip knot around the terminal ileum immediately anterior to the mesoappendix. This served to partially obstruct the flow of chyme. A 10 ml sample of S. flexneri M4243 was injected directly into the lumen of the jejunum. The incision was closed in 2 layers and one end of the slip knotted tape was brought out through the incision. Two hours after the inoculation of bacteria, the slip knot was released. Temperatures, cultures and white blood counts were performed as in the oral challenge rabbits. Histologic sections and cultures were obtained at time of sacrifice.

Mononuclear Cell Isolation. At time of sacrifice, the rabbits from various immunization groups had peripheral blood, Peyer's patches, mesenteric lymph nodes, spleen and axillary lymph nodes removed under asceptic conditions. For the peripheral blood, the buffy coat was placed on lymphocyte separation medium and centrifuged at 400 x g at room temperature for 30 minutes. The cells at the interface were removed, characterized as described below and used as the mononuclear peripheral blood preparations. Tissues were cut into 1 cm square fragments with a sterile blade and placed on sterile wire mesh. The cells were carefully teased apart and passed through the mesh. This material was centrifuged at 400 x g at room temperature for 7 minutes. The pellet was gently resuspended and washed twice in RPMI 1640. The total cells and viability were determined. A Wright stain preparation was examined to determine the differential of the isolated cells.

Phenotyping of Mononuclear Cells. Mononuclear preparations were washed with warm 37 C PBS to remove adsorbed immunoglobulin. They were resuspended in 1ml of a working latex bead solution and incubated for 2 hours at 37 C to detect phagocytic cells. B cells were determined by incubating the latex-treated cells with either 100 ul of a 1:20 dilution of FITC-sheep anti-rabbit immunoglobulin (polyvalent). Following a 30 minute incubation in an ice bath, the cells were washed three times with cold PBS (including 0.1% azide), centrifuged and examined under a Leitz microscope equipped for simultaneous epifluorescence and phase contrast study. The percentage of surface immunoglobulin-bearing and phagocytic cells were enumerated. Cytoplasmic immunoglobulin was determined on air-dried preparations of the mononuclear cells using the same reagents.

Mononuclear Cell Cultures. All cell suspensions were adjusted to 40,000 viable cells/ml. Three hundred ul of cell suspension was added to each of 24 wells in a sterile flat bottomed 96 well microtiter plate. The plates were incubated at 37 C in a 5% CO2 95% air atmosphere. At days 0, 4, 7, 14, and 21, the supernatant was removed from 3 consecutive wells. An aliquot was assayed by the microelisa technique for LPS-specific IgG and IgA.

Paneth Cell Isolation and Tissue Culture. Chronic rabbit ileal loops with an initial length of 20 cm isolated for at least two weeks were used as the source of Paneth cells. Following removal of the isolated loop, pincers were used to invert the bowel. Tissue paper was used to blot the mucosal surface in an attempt to remove most of the adherent mucus. The segment is cut into 3 cm lengths and placed into a trypsinizing flask containing 30 ml of Hank's balanced salt solution (HBSS), pH 7.0, and agitated for 15 minutes with a rotary bar to further remove mucus and possible soluble trypsin inhibitors. Buffered trypsin with antibiotics (BTA) were used to trypsinize the bowel for 3 hours. The trypsinized suspension of cells was filtered first through sterile gauze once to remove mucus and large aggregates. Then, the cells were centrifuged at 300 x g for 10 minutes, and the pellet was resuspended in 15 ml of RPMI 1640 with L-glutamine (Gibco) containing 10% fetal calf serum, 1% antibiotic-antimycotic mixture and 10 ug/ml gentamycin. Three ml of cell suspension were placed into each of 5 sterile 35 mm plastic tissue culture dishes. Placement of sterile glass coverslips on the bottom of the culture dish allowed attachment of cells for ease in later fixation and staining. Different staining techniques were employed to distinguish the Paneth cells from the other epithelial and mononuclear leukocytes in the culture.

Results

I. Role of Adjuvants in Enhancing the Primary Secretory IgA Response of the Intestine.

A. Parenteral Adjuvant Studies. Previous studies in our laboratory have indicated that a small primary local IgA response is detectable in secretions from the isolated ileal loops within a week after stimulation by live, oral Shigella X16 (2,16). Parenteral immunization without adjuvant is ineffective in enhancing the local immune response to the antigens we have studied (6). Since the form of antigen is known to be of considerable importance in the development of a mucosal immune response, we have explored the role of parenterally administered heat-killed Shigella X16 in complete Freund's adjuvant in stimulating the mucosal immune response. Dosage schedules are shown in Table 1.

Table 1. Immunization Schedule

Group	Antigen	Route	Day Given
I	Heat-killed Shigella X16	IM	-1
II	Live Shigella X16	oral	0
III	Heat-killed Shigella X16	IM	-1
	Live Shigella X16	oral	0
IV	Live Shigella X16 & Avridine	oral	0
V	Live S. flexneri M4243A1	oral	0
VI	Live S. flexneri M4243A1	oral	-74, -67, -60
	Live S. flexneri M4243A1	oral	0

Oral immunization given via nasogastric tube with the animal lightly anesthetized.

1. Immune responses following parenteral stimulation with heat-killed shigella in complete Freund's adjuvant. Following a single intramuscular injection, the Group I animals all demonstrated a predictable serum IgG anti-shigella LPS response within one week (Table 2). The IgG activity peaked by the third week after immunization. IgA activity paralleled that of IgG. However, the activity

of IgG and IgA in the intestinal secretions from these animals showed only a weak primary IgA and IgG response (figure 1).

Table 2. Serum IgG Activity to Shigella LPS in Rabbits Given Heat-Killed Shigella Intramuscularly in CFA.

Days Post-immunization	Given Oral Dose *	No Oral Dose *
0	.026 (.016-.044)	.015 (.007-.032)
6-7	.512 (.497-.547)	.243 (.120-.493)
8-14	.804 (.762-.847)	1.29 (1.05-1.60)
15-21	.873 (.817-.933)	1.10 (.899-1.35)
22-28	.914 (.841-.993)	1.94 (1.61-2.34)
29-35	.918 (.811-1.04)	1.73 (1.38-2.17)
36-42	.977 (.929-1.03)	1.69 (1.29-2.22)

* Activity is expressed as Geometric mean with the variance in parenthesis (see Methods section).

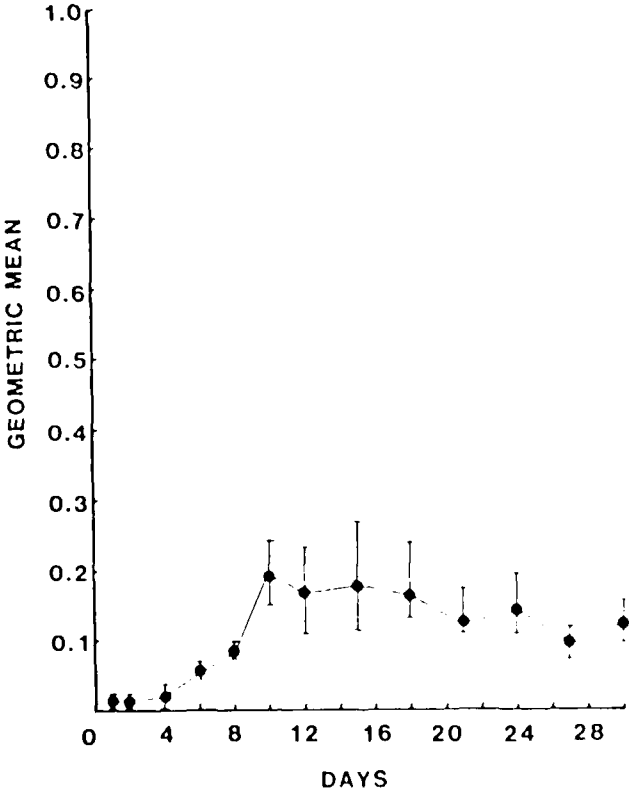

Figure 1. IgA anti-shigella LPS activity in loop secretions from Group I rabbits. Only a weak primary IgA response is elicited following heat-killed shigella in CFA given IM.

2. Immune responses following oral stimulation with live Shigella X16. Group 2 rabbits showed the typical weak primary IgA response in intestinal secretions following the single oral dose of shigella (figure 2). As is typical of this type of stimulation, no IgG anti-shigella LPS activity was detected in intestinal secretions and no IgG or IgA activity was present in the serum.

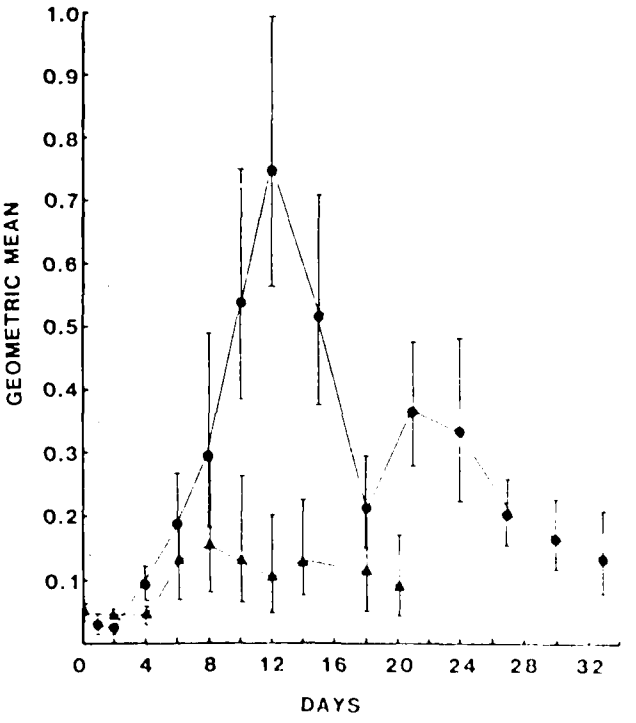

Figure 2. IgA anti-shigella LPS activity in intestinal secretions from animals given only a single oral dose of live Shigella X16 on day 0 (triangles) or animals given a single parenteral dose of heat-killed shigella X16 on day -1 followed by a single oral dose of live Shigella X16 on day 0. By day 8 a significantly greater ($p<.01$) response was seen in secretions from the animals given the combined regimen.

3. Immune responses following combined parenteral immunization with heat-killed Shigella X16 in complete Freund's adjuvant and oral immunization with live Shigella X16. The serum IgG activity was significantly lower at all data points after two weeks in Group III as opposed to the Group I rabbits. In addition, an extraordinarily rapid increase was seen in the IgA anti-shigella LPS activity found in the intestinal secretions of all rabbits in Group III (figure 2). By day 8 there was a significant difference between the animals given only a single oral dose (Group I) and the Group III rabbits in the local IgA activity (figure 2). The local IgA response peaked for Group III intestinal secretions by day 12. It is likely that the B lymphoblasts are stimulated by the strong parenteral stimulation of the antigen with adjuvant while the local stimulation may direct and/or switch those B lymphoblasts to preferentially produce IgA. This hypothesis would also explain the decrease in IgG detected in the serum of these animals. The cellular immune response studies outlined below will be used to address this issue. By determining the means to manipulate the mucosal immune system to the desired response, we can optimize the chances of success for clinical vaccines against enteropathogens.

B. Mucosal Adjuvant Studies. Recent publications indicated that Avridine (N,N-dioctadecyl-N',N'-[2-hydroxy-methyl] propanediamine) could increase immune responses to sheep erythrocytes, influenza B and equine encephalitis virus (21). Careful studies by Anderson demonstrate that such adjuvants as Avridine and CFA may function by increasing lymphoid traffic and inducing angiogenesis with differnetiation of high endothelial venules (22). Although CFA would be unsuitable for use in the lumen of the gastrointestinal tract, we looked at the ability of our system to detect any enhancement of the mucosal immune response to shigella LPS by Avridine. For these studies, a group of 10 rabbits were given a single oral dose of live shigella (Group IV) mixed with Avridine as shown in Table 1. Intestinal secretions from these rabbits gave similar local IgA responses to those from animals not given the Avridine (figure 3). No significant enhancement was found at any data point. Serum samples were uniformly negative for anti-shigella LPS activity of either the IgG or IgA class. Therefore, Avridine was not used in further studies.

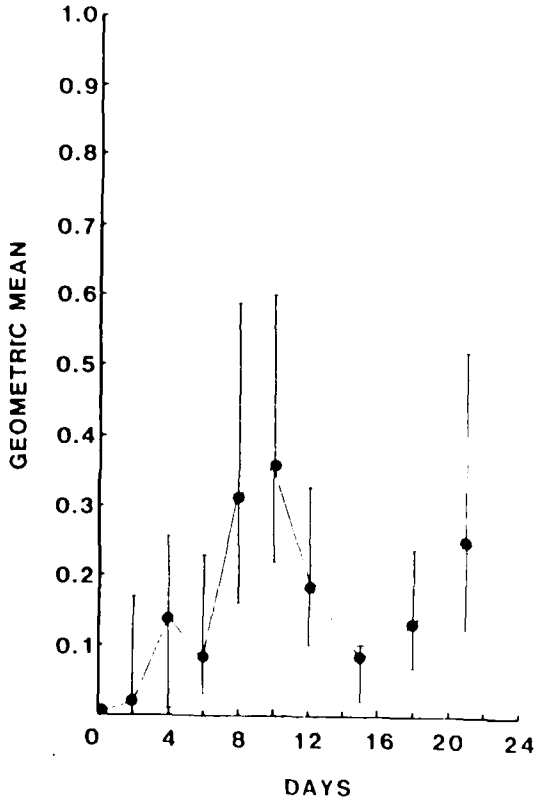

Figure 3. IgA anti-shigella LPS activity in intestinal secretions following a single oral immunization with live Shigella X16 mixed with Avridine. No significant enhancement of the mucosal immune response was found.

II. Role of Antigen Form in the Mucosal Memory Response--Virulence Plasmid.

A. Primary Immune Response to S. flexneri M4243A1. Although previous studies in our laboratory have shown that the noninvasive strain S. flexneri 2457-0 is able to elicit both a vigorous primary and mucosal memory response (17), it possesses a 140 megadalton virulence plasmid. Further, in clinical trials, it has reverted to a pathogenic forms in some case making it unsuitable as a mucosal vaccine. Further, these findings raised the question as to whether in

the experimental situation expression of the virulence plasmid by a few of the bacteria would account for the mucosal memory results obtained. Therefore, in the present studies, a single dose of shigella M4243A1 was given orally on day 0 (Table 1). The resulting intestinal IgA response is shown in figure 4. This strain proved to be highly immunogenic despite the lack of the 140 megadalton virulence plasmid and a negative Sereny test. The secretory IgA levels produced were significantly greater than those seen with the single dose of the locally invasive Shigella X16 strain. As with the other studies, the local IgG response was trivial and systemic IgG and IgA against shigella were lacking.

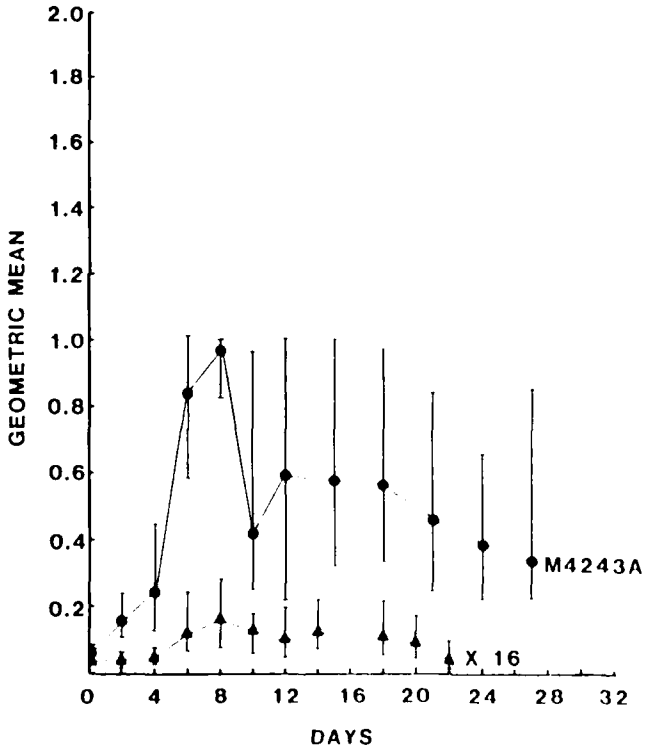

Figure 4. Comparison of IgA anti-shigella LPS in secretions from animals given a single oral dose of live Shigella X16 (triangles) and those given M4243A1 (circles).

B. IgA Memory Response to S. flexneri M4243A1. For the memory response studies, the same dosage (Table 1) was used as in previous memory studies performed with the invasive and noninvasive strains. As shown in figure 5 an impressive secretory IgA memory response was found in the animals given this regimen. A residual secretory IgA response remained even after the 60 days of rest since the last oral dose with this antigen. Further, following the oral challenge dose on day 0, a striking rise was seen in the secretory IgA response. These responses were significantly greater than those of the response elicited by a single dose of the invasive Shigella X16 on all days tested and greater than the primary response to the M4243A1 on all days except 2 an 6-10 where the large standard errors due to the outbred samples prevented significance by the T test. These findings indicate that noninvasive shigella can be effective mucosal immunogens. Furthermore, the 140 megadalton virulence plasmid is irrelevant to the establishment of a primary and secondary secretory IgA response to shigella.

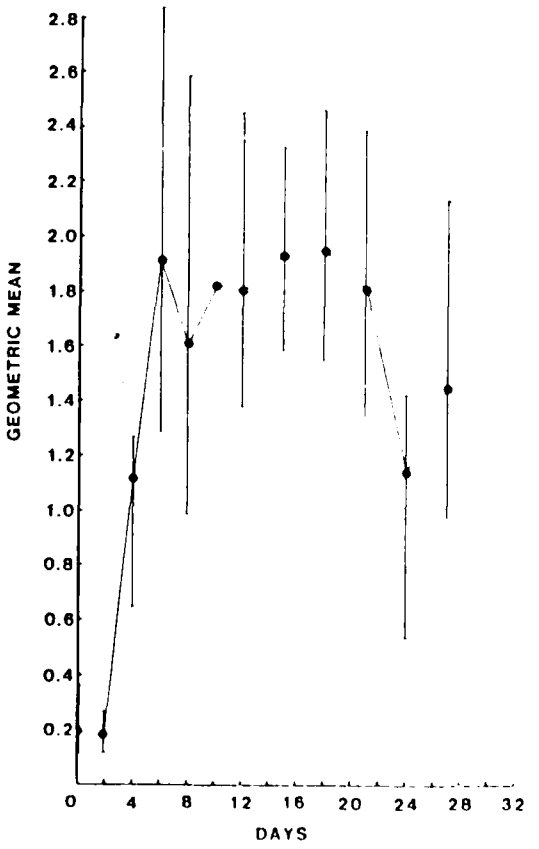

Figure 5. IgA memory response from Group VI.

III. Establishment of Cell Culture for Following Mucosal Stimulation by Enteropathogens.

A. Phenotypes of mononuclear cell populations. As shown in Table 3, most of the isolated mononuclear cell preparations had 20% B lymphocytes. Peyer's patches contain few plasma cells and an important control on contamination of this population by plasma cells in the adjacent lamina propria was the determination of cytoplasmic immunoglobulin-containing cells. As shown in Table 3, only 1% of Peyer's patch mononuclear cells were plasma cells in our preparations.

B. Kinetics of In Vitro Production of IgG and IgA to S. flexneri LPS. Animals from Group I and Group III (Table 1) were used to study the kinetics of the IgG and IgA activity in supernatants from the mononuclear cell cultures. They were chosen to maximize the chance of finding both IgG and IgA activity in these early studies. For both groups, most culture supernatants were negative until day 4 and most peaked by day 8. Data from the peak value for each tissue was chosen to represent the maximal capability of their B lymphocytes to mature to antigen-specific plasma cells. Representative data from the Peyer's patches and spleen from a Group III rabbit (Table 1) is shown in figure 6.

We have begun to use this system to follow the cellular immune responses in rabbits given the immunizing regimens described in Table 1. Some trends are already apparent from the preliminary data available at this time. Parenteral immunization with heat-killed shigella in complete Freund's adjuvant is able to stimulate B cells which will differentiate into both IgG and IgA secreting cells with activity to S. flexneri LPS (Tables 2 and 3). These cells are found in most of the tissues examined by the eighth day after antigen administration. The major exception is the appendix where the lymphocytes produce only a marginal IgG and no detectable IgA response. The strongest IgG responses were found in the peripheral blood lymphocytes and the spleen. Interestingly, the spleen gave a stronger IgA response on day 8 than the marginal response found in the peripheral blood. This finding would be consistent with the notion that IgA precursor B cells mature in the spleen following stimulation. For those days where we have multiple data points, we have noted considerable variation from one animal to another. This is consistent with similar observations on the humoral immune responses studied in this outbred species. Studies on many more animals are underway to allow us to do a proper statistical evaluation of the development on IgA and IgG antigen-specific cells following the various modes of stimulation which we have established with our intestinal loop model system. Further, we suggest the use of inbred mouse strains to more carefully identify the details of stimulation of antigen-specific cells.

Table 3

TISSUE MONONUCLEAR CELL TYPE DISTRIBUTION

TISSUE SOURCE[a]	CELL TYPE[b]	% AVERAGE[c]	+/- 2 S.D.	N
PERIPHERAL	SIg+	32	20	17
BLOOD	CIg+	1	3	16
	PHAGO.	9	15	17
PEYER'S	SIg+	17	16	18
PATCH	CIg+	0.5	1	17
	PHAGO.	11	11	18
MESENTERIC	SIg+	19	26	18
LYMPH NODE	CIg+	1	3	16
	PHAGO.	6	8	18
AXILLARY	SIg+	12	16	18
LYMPH NODE	CIg+	0.5	2	15
	PHAGO.	7	8	18
BRONCHIAL	SIg+	12	10	18
LYMPH NODE	CIg+	2	8	18
	PHAGO.	7	9	18
SPLEEN	SIg+	25	14	18
	CIg+	0.6	2	18
	PHAGO.	14	10	18
APPENDIX	SIg+	6	9	18
	CIg+	0.9	2	16
	PHAGO.	6	7	18

a: Tissue mononuclears were isolated as described in the methods. These results include all model types. No significant differences in overall tissue mononuclear types were seen between the models (data not shown).

b: SIg+ = surface immunoglobulin positive; CIg+ = cytoplasmic immunoglobulin positive; PHAGO. = phagocytosis of latex beads. See methods for further details.

c: Percentages were estimated from 100 to 300 cell counts.

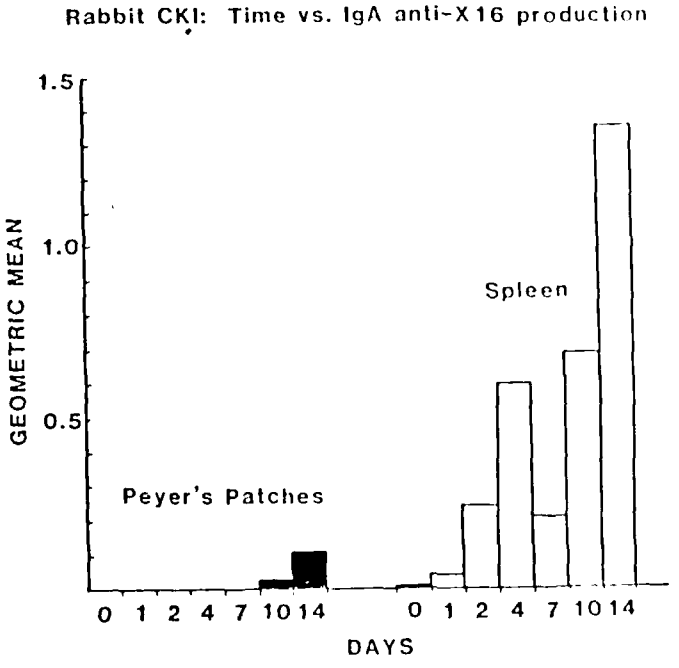

Figure 6. IgA anti-shigella LPS production from Peyer's patch and spleen cells cultured for the indicated number of days.

IV. Shigella Challenge Studies.

A. In Vitro Studies to Follow Shigella Adhesion and Invasion.

1. Adhesion to colonic and ileal tissue sections. Two major problems were found with the intact tissue section system. First, histologic studies of the sections revealed that the S. flexneri M4243 infrequently adhered to the epithelium and was not found attached to the serosa. In later studies, we have noted that the shigella are taken-up by the specialized epithelium overlying Peyer's patches and isolated lymphoid follicles (see section V). However, this represented only a small number of the bacteria within the broth. A dose response study revealed that when 10 million

or more bacteria are included in the culture containing no serum or intestinal secretions, less than 1 % will adhere to the tissue sections. Thus, in any attempted protection studies with antigen specific IgA, we would be forced to measure minor differences. Because of this, we chose to establish a tissue culture line of cells to increase the percentage of bacteria adhering and the percentage of epithelium involved.

2. Attachment and invasion into HeLa cells. The HeLa cell system proved to be much more reliable in terms of generating consistent numbers of infected cells. Using the conditions outlined in the Methods section, an average of 54.1% of HeLa cells were involved by more than 10 bacteria per cell while an average of 4.5% had no attached bacteria (Table 4). When normal rabbit serum was incubated together with the S. flexneri M4243, there was a significant inhibition of the involvement of HeLa cells by bacteria. In this group, only 26.5% of HeLa cells were involved by more than 10 bacteria while an average of 24.4% had no involvement. Similarly, when immune serum to M4243 was used, only 25.8% of the HeLa cells were involved by more than 10 bacteria and an average of 49.1% of HeLa cells were totally uninvolved. This indicated that there was an inhibitory influence of serum on this attachment. However, there were significantly more uninfected cells in the group treated with immune serum vs. normal serum (P<.01). This system is now being used to study the effects of intestinal secretions from immunized and unimmunized rabbits on the attachment of bacteria.

Table 4. Shigella Adherence to HeLa Cells.

n=9	* Number of Bacteria Adhering to HeLa Cells				
Serum	0	1	2-5	6-10	>10
None	4.5(3.4)	4.3(4.7)	16.7(10.2)	20.2(10.7)	54.1(20.4)
Cont	24.4(19.4)	10.9(10.7)	19.9(9.7)	18.3(20.2)	26.5(24.0)
Immun	49.1(20.6)	7.6(5.5)	12.0(7.9)	5.7(4.5)	25.8(11.0)

* Data expressed as percentage of HeLa cells containing the indicated number of bacteria (standard deviation indicated).

B. In Vivo Studies to Follow the Pathologic Effects of S. flexneri.

1. Oral challenge to intact rabbits. The groups of rabbits used in this study are shown in Table 5. To follow the effects of oral challenge by S. flexneri M4243 to intact rabbits, we first determined the effect of different dosages on temperature, peripheral blood leukocytes count and differential (Table 6 and 7). As shown in Tables 6 and 7, there was little difference in the temperatures or leukocyte counts of the animals following challenge with shigella on day 0. Notable exceptions included the animals given opium to decrease gastrointestinal motility. These animals (groups III-VI) all had significant decreases in temperature in the afternoon 4 hours after the oral challenge. This is due to the effect of the opium and not to infection. Similarly, no difference was found in the leukocyte differential from one group to another. These findings indicate that rectal temperature and leukocyte studies will be inadequate as an indicator of disease in intact animals.

Table 5. Groups for Oral Challenge with Shigella.

Group	Regimen for Challenge
I	10 ml .1N bicarbonate, 10 ml BHI
II	10 ml .1N bicarbonate, 10 ml of 10^9 Shigella
III	5 ml .2N bicarbonate, 10 ml opium, 10 ml BHI
IV	5 ml .2N bicarbonate, 10 ml opium, 10 ml of 10^8 Shigella
V	5 ml .2N bicarbonate, 10 ml opium, 10 ml of 10^{10} Shigella
VI	5 ml .2N bicarbonate, 10 ml opium, 10 ml of 10^{11} Shigella

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Dosage given under light anesthesia with xylazine and ketamine. Orogastric tube used for administration of bacteria.

TABLE 6 RECTAL TEMPERATURE

Temps °F	I	II	III	IV	V	VI
Day 1 am	-	102.4 ± .11	-	102.1 ± .15	102.3 ± .14	102.4 ± .10
pm	-	102.3 ± .16	-	102.0 ± .09	102.1 ± .23	102.3 ± .11
Day 0 am	102.9 ± .40	102.4 ± .09	102.7 ± .08	102.3 ± .23	102.1 ± .44	102.3 ± .16
pm	102.7 ± .20	102.3 ± .15	98.4 ± .41	99.1 ± .51	98.5 ± .59	99.2 ± .38
Day 1 am	102.7 ± .14	103.6 ± .17	102.3 ± .10	102.6 ± .11	102.8 ± .37	102.6 ± .17
pm	103.0 ± .17	103.7 ± .24	102.4 ± .21	102.7 ± .32	103.4 ± .67	-
Day 2 am	102.2 ± .17	102.6 ± .09	102.9 ± .21	102.2 ± 0	102.8 ± .34	-
pm	103.2 ± .17	103.2 ± .18	102.6 ± .23	-	102.7 ± .25	-

TABLE 7 LEUKOCYTE COUNTS

WBC X $10^3/MM^3$	I	II	III	IV	V	VI
Day 1	-	9.73 ± .66	-	11.8 ± 1.68	10.7 ± 3.1	9.5 ± .17
Day 0	10.3 ± .50	9.35 ± .89	9.3 ± .54	11.1 ± 1.46	12.48 ± 2.7	10.6 ± 1.11
Day 1	13.4 ± .72	14.56 ± 1.8	16.0 ± 1.1	15.4 ± 2.5	13.14 ± 4.0	16.4 ± 2.75
Day 2	12.5 ± 1.46	16.68 ± 2.5	12.6 ± 1.8	14.9 ± 4.93	11.34 ± 1.3	-
Day 3	12.3 ± .69	12.93 ± 3.6	10.4 ± .92	-	11.75 ± 1.2	-

2. RITARD Model for Shigella Invasion. The RITARD model has been used in our laboratory to control variables which we presumed were affecting the oral challenge system. Although the animals for the oral challenge study were fasted, they always had considerable gastric and intestinal contents. This may have diluted the dose of antigen and even with bicarbonate, the gastric acid and pepsin may have had enough antibacterial activity to prevent invasion. With the RITARD model, the shigella are directly injected into the jejunum thereby avoiding the gastric acid and the considerable dilution effect of lagomorph gastric contents. The temporary ligature around the terminal ileum resulted in only minor adhesions (noted at time of sacrifice).

As shown in Table 8, there was a significant difference ($P<.01$) in the leukocyte count between the day 0 and day 1 values of rabbits given live S. flexneri M4243 as opposed to the rabbits given only broth. The surgical procedure alone, therefore, does not produce such leukocytosis to obscure the clinical effects of the inoculum. The jejunum, ileum and colon from these animals have been examined histologically. By 24 hours, significant inflammation is present in the ileum. The bacteria are present most frequently over the follicular areas of Peyer's patches accompanied by a striking acute inflammatory response. Future studies will use this system to determine the effect of the systemic and mucosal immune response to S. flexneri on these responses.

Table 8. Peripheral Blood Leukocyte Count in RITARD Model.

Innoculum	n	Day 0 *	Day 1	Day 2
Broth only	4	9.0(1.2)	11.2(2.1)	10.1(2.2)
10^8 Shigella	5	8.4(2.1)	12.3(1.8)	10.7(2.9)

* Data is expressed as leukocyte count per cubic mm (standard deviation is indicated).

V. Antigen Processing of Shigella in the Gut-Associated Lymphoid Tissues.

Our previous studies have shown that live preparations of S. flexneri are able to elicit a vigorous primary and mucosal memory response (regardless of the ability to invade), however, heat-killed preparations are unable to prime animals for a mucosal memory response (16). The heat-killed preparations do contain appropriate antigens for stimulating secretory IgA against shigella LPS as when they are injected directly into Peyer's patches, a strong local IgA response results. Dosage effect was studied previously and shown not to be the key factor. Even extraordinarily high doses of heat-killed preparations were ineffective at eliciting a mucosal memory response (17). Recent studies by Owen et al. have suggested that processing of bacteria by the "M" cells which overlie isolated follicles and Peyer's patches in the intestine may differ when the bacteria is alive or dead (23). Therefore, in these studies, we have sought to determine the kinetics of antigen processing by "M" cells and to explore the possible role of phagocytic Paneth cells.

Only preliminary information has been gathered at the present time for this part of the proposal. We have established the technique for acute isolated intestinal loops and are evaluating different preparations of S. flexneri (Methods Section) to determine the time-course of their uptake and the duration of their detection in Peyer's patches. With the M4243 strain, bacteria have been found within the "M" cells as early as 30 minutes after administration of bacteria to the intestine. By 4 hours, many shigella are found within the "M" cells (figure 7), but have not been seen in absorptive epithelial cells over villi or in Paneth cells. The bacteria are packaged into large vescicles (figure 7), but have not been noticeably damaged by the 4 hour period so far examined. Their cell wall is intact and they maintain their rod-shape (figure 8). Ongoing studies will document the kinetics of this uptake for both live and killed shigella. Future studies will deal with the effects of specific and nonspecific IgG and IgA on the processing of antigen. Lastly, the role of "M" cell uptake in the pathogenesis of dysentery is an as yet unexplored area which could explain the focal nature of the lesions seen in human disease.

Figure 7. Electron photomicrograph of an "M" cell overlying a Peyer's patch. The "M" cell has phagocytosed several bacteria and contains them within membrane-lined vescicles. Five of the bacteria are cut in cross-section while the rod-shape is apparent in one. Magnification = 7300 X.

Figure 8. High power electron micrograph showing detail of a shigella within the vescicle of an "M" cell. The cell wall of the bacteria is intact and shows no evidence of dissolution at this time. Magnification = 54,200 X.

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Relevant Collaborative Activity

During this contract period we have been involved in collaborative experiments with several local, national and international groups looking at immune responses to pathogenic microorganisms. Locally, we have worked with the Unit for Laboratory Animal Medicine on a problem common to all workers with experimental animals, Pasteurella multocida infections. Nationally, with Drs. Roth and Owen, we have performed immunohistochemical analysis of human and monkey AIDS tissue infected with Mycobacterium avium-intracellulare. Other studies are in progress with Dr. Morgan on the role of antibody-dependent, cell-mediated cytotoxicity in Shigella sonnei infections. With Dr. Formal, we have reestablished the histopathologic grading system to quantify the lesions in experimental shigella infections. Lastly, we have performed further studies with Dr. Tagliabue's group on the ability of mucosal lymphoid cells to produce antibody-dependent, cell-mediated cytotoxicity against S. flexneri.

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